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Dissociation of interleukin-4 and interleukin-5 production following treatment for *Schistosoma haematobium* infection in humans

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SUMMARY

Infection with Schistosoma haematobium, *the causative agent of urinary schistosomiasis is characterized by high levels of specific immunoglobulin (Ig) E and eosinophilia. The primary cytokines driving production of IgE and eosinophilia are IL-4 and IL-5, respectively. In this study, IL-4 and IL-5 production in children from a schistosome endemic area of Zimbabwe were investigated. Blood samples were taken, stimulated* in vitro *with either mitogen or schistosome antigens and assayed for IL-4 and IL-5 production. These samples produced either IL-4 or IL-5 but rarely both cytokines when blood was cultured* in vitro *for 24 or 48 h. After 72 h culture* in vitro*, both cytokines were detected in most samples. These data imply that while IL-4 and IL-5 are both produced by schistosome infected people, they are not necessarily coproduced.*

Keywords *Th2-subsets,* Schistosoma haematobium, *human*, *interleukin-4*, *interleukin-5*, *Zimbabwe*

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INTRODUCTION

In common with many other helminth infections, urinary schistosomiasis caused by *Schistosoma haematobium* is associated with elevated levels of serum immunoglobulin (Ig)E (Hagan *et al*. 1991) and eosinophilia (Hagan *et al*. 1985). IgE production is strongly associated with interleukin (IL)-4 (Hagan *et al*. 1985, Snapper & Paul 1987) and eosinophilia levels with IL-5 (Sanderson 1992, Sanderson 1993). Both IL-4 and IL-5 are produced by Th2 cells (Jung *et al*. 1995, Kay *et al*. 1995) although these cytokines can also be produced by other cell types (Gibbs *et al*. 1996, Shimizu *et al*. 1998).

Although they are both type-2 cytokines, circumstantial evidence that IL-4 and IL-5 may be independently regulated during schistosomiasis has come from studies of *Schistosoma mansoni* in a Kenyan population where IL-5, but not IL-4, was shown to be a significant determinant for post-treatment infection intensities (Roberts *et al*. 1993). In a study of an endemic focus in Gabon, 2 years after treatment, uninfected people had higher levels of IL-5 than those who became reinfected, whereas there was no difference in IL-4 production between the two groups (Grogan *et al*. 1998). Conversely, IL-4 but not IL-5 production was significantly greater 5 weeks post treatment compared to pretreatment in the same *S. haematobium* focus (Grogan *et al*. 1996). The aim of the current study was to investigate the degree of coregulation of IL-4 and IL-5 in this disease; our evidence supports the hypothesis that these two cytokines may be regulated independently. This hypothesis is supported by intracellular cytokine staining experiments which have reported that IL-4 and IL-5 were produced predominantly by different human peripheral T-cells, in blood from healthy volunteers, from patients with hyper-IgE syndrome (Jung *et al*. 1995) and atopic asthma (Krug *et al*. 1998). Both these studies report cytokine production by cells stimulated with either anti-CD3 or PMA and ionomycin. Unfortunately, flow cytometry is less suited to detect low frequency events in the range

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usually observed in antigen-stimulated cells. We believe that the current study documents a special situation in which the separate regulation of IL-4 and IL-5 can be demonstrated in schistosome antigen-stimulated cells.

MATERIALS AND METHODS

Fifty-five children aged 6–14 years were recruited from two schools in the Burma Valley in the Eastern Highlands of Zimbabwe in August 1994. There were 30 girls and 25 boys. These children were a subset of a cohort used to study humoral responses to *S. haematobium* infection (Mutapi *et al.* 1997). The prevalence of infection was 40·0% before treatment and 5·0% some 9 months after treatment (Kaswa School) and 85·7% before treatment and 28·6% some 9 months after treatment (Valhalla School).

The level of infection was determined for each child in November 1994 and August 1995 by filtration of 10 ml of each of two urine samples collected on different days for *S. haematobium* eggs (Mott 1983). Each child was successfully drug treated in November 1994 with 40 mg/kg praziquantel. All children in the study were free of *S. mansoni* infection as determined by Kato–Katz preparations from stool samples (Katz *et al.* 1972).

In November 1995, 10 ml of blood was taken from each child, transported from the study site to Harare and used for cytokine studies. A whole blood culture method was used because it permitted us to study cytokine production in an environment that reflected the in-vivo situation better than cultures of isolated peripheral blood mononuclear cells (PBMCs) (De Groote *et al.* 1999, Zangerle *et al.* 1999); minimal manipulation was necessary for the preparation of samples and all cell types were present preserving normal cell to cell interactions. The use of autologous plasma, where circulating stimulatory and inhibitory mediators are more likely to reflect in-vivo proportions, avoids potential skewing of the immune response by heterologous plasma (De Groote *et al.* 1999). Whole blood culture techniques have been successfully used by a number of other groups (Meyaard *et al.* 1997, Zangerle *et al.* 1999).

A total of 500 μl of blood was diluted with 500 μl of RPMI 1640 supplemented with 2·4 mM sodium hydrogen carbonate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 × nonessential amino acids (Gibco, Paisley, UK, 11140–035), 1 mM sodium pyruvate and 50 M β-mercaptoethanol. Cultures were incubated in flat bottomed 24-well culture plates (Corning Costar, High Wycombe, UK) in a humidified environment at 37°C and 5% CO_2 in the presence of 10 μg/ml of either: phytohaemagglutinin (PHA) (Sigma Cat. No. L4144, Sigma, St Loius, MO, USA), schistosome egg antigens (SEA), adult worm antigens (AWA) or incubated without stimulant. *S. haematobium* antigens were purchased in freeze dried form from the Schistosome Biological Supply Programme (Theodore Bilharz Research Institute, Giza, Egypt). They were reconstituted in sterile phosphate buffered saline. 24, 48 or 72 h post incubation, culture supernatants were harvested and stored at − 20°C. All of the samples contained viable cells as demonstrated by their production of other cytokines (GM-CSF, IL-10, IFN-δ or TNF-α) (data not shown).

Because limited volumes of blood were available, not all samples provided supernatants at all three time points. However, for each stimulant (PHA, SEA or AWA) results from the same children have been reported for the 24 and 48 h time points. Of the 55 children, data exist for 39 PHA, 41 SEA and 52 AWA stimulated cultures. Results from a smaller subset have been reported for the 72 h time point.

IL-4 and IL-5 were detected in culture supernatants using sandwich ELISAs, utilizing antibody pairs (Pharmingen, Erembodegem-Aalst, Belgium) (protocol available on request). The ELISAs had a sensitivity of 0·0625 U/ml and an accuracy of 0·01 U/ml. A standard curve was run on each plate using recombinant human IL-4: NIBSC 88/656 I.S. [1000 international units (IU) per ampoule] or IL-5: NIBSC 90/586 R.R. [5000 units (U) per ampoule] as appropriate. A sample was classified as positive if it produced over 0·02 U/ml of net cytokine (cytokine produced on stimulation with antigen or mitogen minus spontaneous cytokine production).

Statistical analysis

Data were analysed using statistical software (SPSS for Windows version 6, Chicago, IL, USA). Spontaneous IL-4 and IL-5 production was subtracted from all values. Hence, some values became negative and the presence of these spurious negative values gives an indication of the stochastic variability in our data set, which is clearly within an acceptable range. For example in Figure 1(a), only three negative values are observed. The analyses presented have been repeated using the levels of IL-4 and IL-5 production without these 'background' levels removed, and the results are unchanged (data not shown).

Chi-squared was used to investigate the hypothesis that children produced either IL-4 or IL-5 but not both, on stimulation with the same antigen or mitogen and incubated for the same length of time. Children who did not produce detectable amounts of both cytokines were not included in the chi-squared analysis to avoid false positive results. The statistical treatment used categorical data and therefore did not take account of the levels of cytokines that were produced, but information on levels of cytokine production is shown in the accompanying scatter graphs.

Additionally, logistic regression analysis was used to ascertain whether there were differences between IL-4

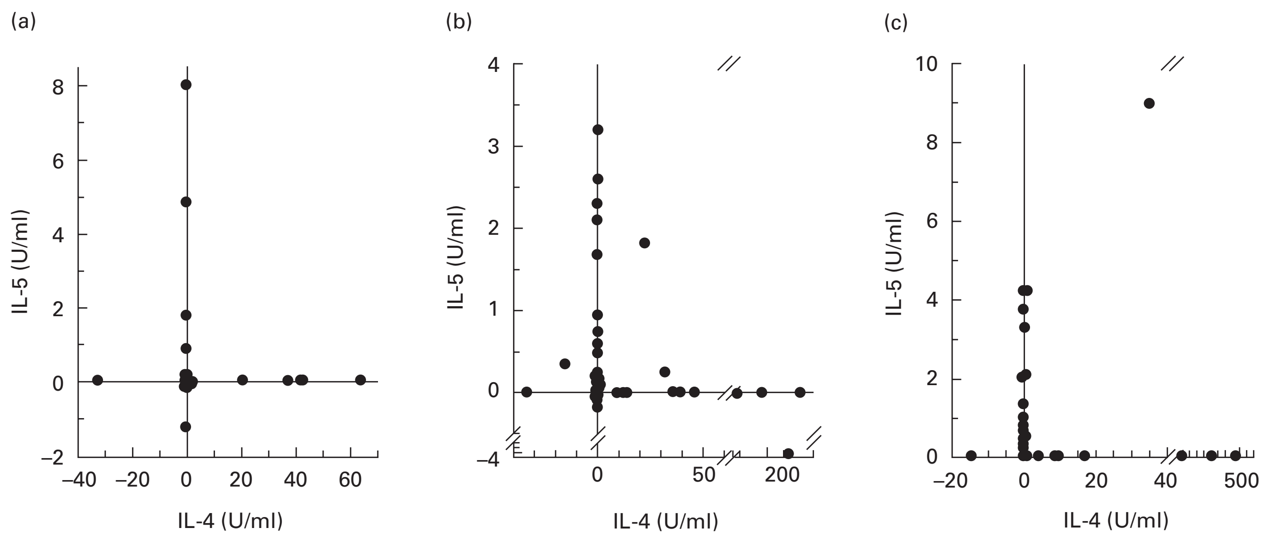
producers compared with nonproducers and IL-5 producers compared with nonproducers. The forward stepwise approach to model fitting was used with five categorical independent variables: school, sex, age, pretreatment infection status and infection status 8 months after treatment. A minimum sample size of 25 was considered appropriate since five independent variables were considered. Cytokine data were first regressed against the independent variable that explained variation in cytokine data with the highest level of significance. The explanatory independent variable was subsequently entered into the model after all other independent variables. If it could still significantly explain variation in cytokine data, it was accepted.

For each combination of cytokine and antigen, there were data resulting from the three time points. This allowed three opportunities to obtain a significant result, so the significance values were corrected to account for multiple tests using a sequential Bonferroni technique (Rice 1989).

Differences between levels of cytokine produced at 24, 48 or 72 h post incubation were tested using Wilcoxon signed ranks tests. The difference between 24 h and 48 h, 24 h and 72 h and 48 and 72 h were tested separately. In each case, the result was accepted if the total sample size was greater than 10. In order to correct for the multiple tests, the level of significance was set using a sequential Bonferroni technique (Rice 1989).

RESULTS

The relative production of IL-4 and IL-5 in samples from individual children after *in vitro* culture for 24 h is shown in Figure 1. If children produced both cytokines, as might be



(d)

Stimulant	Number of children producing				Chi-squared	Degrees of freedom	*P*
	No IL-4 or IL-5	IL-4 only	IL-5 only	Both IL-4 and IL-5			
SEA	30	8	5	0	–	–	–
AWA	27	10	13	4	13·37	1	0·0001
PHA	6	10	17	6	13·36	1	0·0001

–, Chi-squared test not appropriate because no samples produced both IL-4 and IL-5.

Figure 1 A comparison of the levels of production of IL-4 and IL-5 detected by ELISA after in-vitro culture of blood samples from Zimbabwean children for 24 hours in the presence of *S. haematobium* egg antigen (SEA) (a), schistosome adult worms (AWA) (b) and the mitogen, PHA (c). Background levels of cytokine production in the absence of stimulant have been subtracted, hence negative values were observed in a few samples. Each point on the graphs is from a separate blood sample and near the intersection of the axes there is some overlap of points because of those blood samples negative for both cytokines. The associated table shows the number of samples producing IL-4, IL-5, both cytokines or neither cytokine. Statistical comparisons shown are between the number of samples producing either IL-4 or IL-5 and the number producing both cytokines. This analysis shows that blood from more children produced either IL-4 or IL-5 than produced both cytokines on stimulation with SEA, AWA and PHA, 24 h post incubation.

predicted by a 'classical' Th2-like response, their respective data points would fall in the upper right hand quadrant of each graph. It is clear that most data points lie along either axis, indicating that children generally produced either IL-4 or IL-5, but not both and this pattern is evident irrespective of the stimulant used. The associated table indicates that the blood of at least four times as many children produced either IL-4 or IL-5 as produced both, and a comparison of single cytokine versus dual cytokine production is statistically significant (SEA, chi-squared not appropriate because no samples contained both IL4 and IL-5; AWA, $P < 0{\cdot}0001$, PHA, $P < 0{\cdot}0001$).

Forty-eight hours after culture *in vitro*, the results were essentially the same to those at 24 h. This result was statistically significant for the use of both antigen preparations [SEA, $P < 0{\cdot}001$; AWA, $P < 0{\cdot}02$ (data not shown)]. The results using PHA show a similar trend, but were not statistically significant; however, when an individual produced both cytokines, one cytokine predominated (data not shown).

After 72 h of culture *in vitro*, blood no longer tended to produce one cytokine or the other. There was no significant difference in the number of children whose blood produced either IL-4 or IL-5 compared to the number of children whose blood produced both cytokines on stimulation with either AWA or PHA. A similar trend was apparent for the samples cultured with SEA, but the small sample size prohibited statistical analysis.

One potential explanation for the dissociation of IL-4 and IL-5 production after 24 and 48 h in culture is that the kinetics of production are very different for the two cytokines. We therefore investigated kinetics of production. The results are shown in Figure 2 and the statistical analysis of these data is given in Table 1. On stimulation with SEA, there was no significant difference between levels of IL-4 or IL-5 produced between any of the time points (Figure 2a,b). When blood was cultured with AWA, there was no significant difference between IL-4 production between any of the time points (Figure 2c), but IL-5 production after 72 h was higher than after 24 h ($P < 0{\cdot}05$, Figure 2d). Conversely, on incubation with PHA, there was no significant difference in production of IL-5 with time (Figure 2f), whereas IL-4 production peaked at 72 h postincubation ($P < 0{\cdot}01$, Figure 2e). These data indicate that the kinetics of IL-4 and IL-5 production alone cannot explain the observed dissociated production of these two cytokines.

The capacity to generate an IL-4 and/or IL-5 response could arise because the blood sample was from an individual infected with *S. haematobium* or immune to this parasite. This study design permitted us to distinguish between these possibilities because all children were given antischistosomaisis treatment 1 year before the cytokine responses were measured and this treatment allows the development of a protective-type of immune response, as determined by changes in antibody isotype/subclass profiles (Mutapi *et al.* 1998). We therefore investigated whether harbouring a schistosome infection before or after treatment determined if IL-4 and/or IL-5 was produced. Infection status 9 months after treatment did not explain any variability in IL-4 or IL-5 production (results not shown). However, pretreatment infection status was a significant explanatory variable for IL-4 production when cultured with SEA for 24 h ($B = -1{\cdot}4095$, $P < 0{\cdot}05$) and for IL-5 when cultured with PHA for 24 h ($B = -1{\cdot}1466$, $P < 0{\cdot}05$). These data show that the blood from 75% of children who were infected before treatment produced IL-5 on incubation with PHA for 24 h, compared to 40% of blood from children who were not infected. Conversely, blood from 8·1% of children who were infected before treatment produced IL-4 on incubation with SEA compared to 42% of blood from children who were uninfected before treatment.

DISCUSSION

IL-4 and IL-5 were rarely coexpressed when blood from the same child was cultured for 24 h with SEA, AWA or PHA or for 48 h with AWA or SEA. Although not statistically significant, this trend was evident at 48 h when samples were stimulated with PHA. Separate cell populations could have expressed the two cytokines; a hypothesis supported by intracellular cytokine staining experiments (Jung *et al.* 1995, Krug *et al.* 1998). A model splitting the Th2 population into IL-4+/IL-5+, IL-4+/IL-5− and IL-4−/IL-5+, has previously been discussed by Sewell & Mu (1996), and a possible mechanism described by Zhang and colleagues. They reported that IL-5 gene expression can be induced by ectopic expression of the transcription factor, GATA-3, without driving IL-4 gene expression (Zhang *et al.* 1999).

After culture for 72 h, blood from a significant proportion of children produced both IL-4 and IL-5 on stimulation with AWA and PHA. This could be interpreted in two ways. Cells that had previously produced either only IL-4 or only IL-5 could have begun to coexpress both cytokines by 72 h of incubation, or separate cell types may have produced the different cytokines but were activated sequentially. The experimental design could not distinguish between these alternatives.

Fresh human T-cells have been induced to coexpress IL-4 and IL-5 by repeated stimulation (Jung *et al.* 1995), supporting the coexpression scenario. However, Jung and colleagues (Jung *et al.* 1995) cultured fresh PBMCs for 14 days before observing coexpression of IL-4 and IL-5 in a significant proportion of cells. This implies that the 72-h culture protocol used in the current study, with antigen

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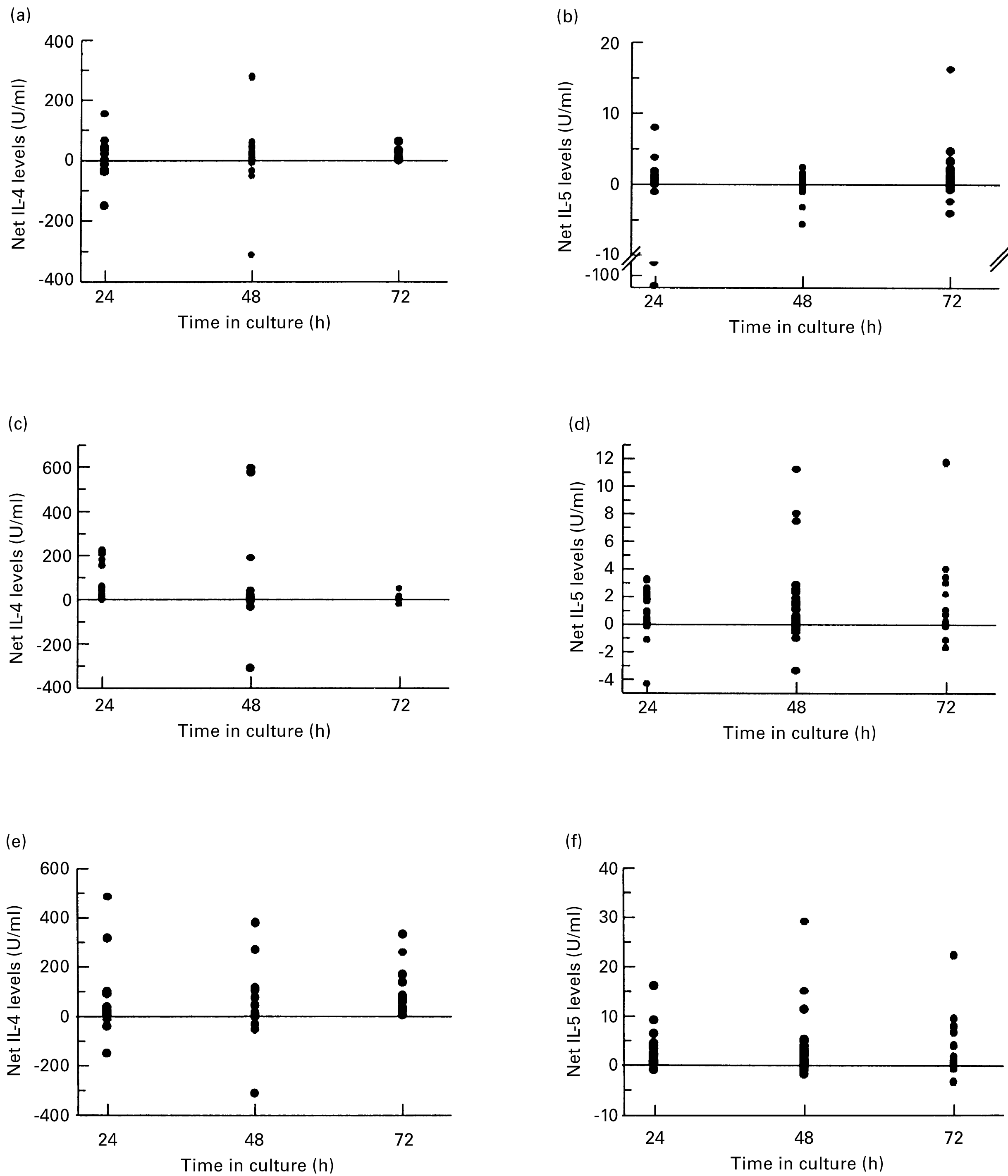

Figure 2 Time course of IL-4 and IL-5 production by blood samples from Zimbabwean children cultured *in vitro*. Cytokine levels were detected by ELISA in response to SEA (a,b), AWA (c,d) or PHA (e,f). Background levels of production in the absence of stimulant have been subtracted, hence negative values were observed in a few samples.

Table 1 A comparison of levels of cytokine measured in supernatants collected at different time points after stimulation with Schistosome egg antigen (SEA), adult worm antigen (AWA) or phytohaemagglutinin (PHA). Wilcoxon signed rank tests were used to make the comparisons shown.

		24 versus 48 h			48 versus 72 h			24 versus 72 h		
Cytokine	Stimulant	N	Z	*P*	N	Z	*P*	N	Z	*P*
IL-4	SEA	47	−0·216	0·829	17	−1·376	0·169	16	−0·384	0·701
IL-5	SEA	45	−0·225	0·822	14	−1·334	0·182	12	−1·362	0·173
IL-4	AWA	58	−0·181	0·856	25	−0·724	0·469	23	−0·747	0·455
IL-5	AWA	54	−0·827	0·068	16	−0·628	0·530	22	−2·539	0·011[a]
IL-4	PHA	46	−2·065	0·039[b]	18	−1·706	0·088	16	−2·953	0·003[c]
IL-5	PHA	39	−1·831	0·067	14	−0·094	0·925	15	−1·761	0·078

P values are given in the table before correcting for multiple tests [a]Statistically significant ($P < 0{\cdot}05$) after correcting for multiple tests using a sequential Bonferroni technique. [b]Not statistically significant ($P < 0{\cdot}05$) after correcting for multiple tests. [c]Statistically significant ($P < 0{\cdot}01$) after correcting for multiple tests.

added only once, may have been insufficient to stimulate coexpression in the same manner.

IL-4 and IL-5 are independently regulated in other disease systems including atopic dermatitis (Kagi *et al*. 1994), asthma (Walker *et al*. 1992, Krug *et al*. 1998) and psoriasis (Vollmer *et al*. 1994). IL-5 is associated with eosinophilia (Sanderson 1992, Sanderson 1993), whereas IL-4 is associated with IgE production (Snapper & Paul 1987, Defrance *et al*. 1987, Finkelman *et al*. 1990, King *et al*. 1990) and this is reflected in the resulting eosinophil or IgE levels when one of these cytokines is produced in the absence of the other (Walker *et al*. 1992, Kagi *et al*. 1994, Vollmer *et al*. 1994, Krug *et al*. 1998).

Mahanty and colleagues, in two studies of in-vitro cytokine production by PBMCs from helminth infected people, observed that concentrations of IL-4 and IL-5 measured in culture supernatants were significantly correlated, as were the frequencies of IL-4 and IL-5 producing cells (Mahanty *et al*. 1992, 1993). However, in the second study (Mahanty *et al*. 1993), they reported that frequency of IL-4 producing cells differed from that of cells producing IL-5; more than double the percentage of cells secreted IL-4 compared to IL-5 when cultured with parasite antigen. Medhat *et al*. (1998), in a study of *S. haematobium*, reported no correlation between the frequency of IL-4 and IL-5 producing cells in subjects reported to be either susceptible or resistant to infection. They also reported that the frequencies of IL-5-producing cells were 5–10 fold greater than those of IL-4. Collectively, these studies imply that parasite antigen does not simply switch on Th2 cells, which then produce the whole spectrum of Th2-type cytokines, but rather that IL-4 and IL-5 can be produced independently of each other.

In a re-infection study of *S. haematobium* infection, Grogan and colleagues, observed that IL-4 secretion by PBMC from infected people (both children and adults) increased 5 weeks after treatment, but there was no change in levels of IL-5 (Grogan *et al*. 1996). These authors attributed this change to either the lifting of an adult worm-induced Th2 suppression or the stimulation of IL-4+/IL-5− cells but not IL-4−/IL-5+ cells, by antigen released as a result of praziquantel treatment. In the same cohort 2 years after treatment, uninfected people had higher levels of IL-5 than those who became reinfected, whereas there was no difference in IL-4 production between the two groups, suggesting at this stage that IL-4+/IL-5− cells were unaffected by a schistosome-induced suppression of IL-4−/IL-5+ cells (Grogan *et al*. 1998). These results suggest that IL-4 can be suppressed (or induced) without affecting IL-5 and *visa versa*.

In the current study, IL-5 produced on stimulation with PHA was associated with infection before praziquantel treatment, whereas IL-4 produced on stimulation with SEA was associated with absence of infection. This could imply that the capacity to produce IL-5 was associated with a history of infection and SEA-induced IL-4 production was associated with resistance to infection. If IL-4 and IL-5 producing cells are of two types, IL-4+/IL-5− and IL-4−/IL-5+, then this might imply that treatment of infected children resulted in the dominance of a IL-4−/IL-5+ cell type whereas in children who were uninfected before treatment, the IL-4+/IL-5− cell type was already dominant. That treatment of infected children affects their subsequent immune response is supported by observations of circulating antibody levels in the same population of Zimbabwean children (Mutapi *et al*. 1998).

The current study and those of Grogan and colleagues (Grogan *et al*. 1996, 1998) illustrate that IL-4 and IL-5 are not necessarily produced in parallel in response to schistosome antigens, as the classical Th1/Th2 model implies (Abbas *et al*. 1996). If this is correct, the source of cytokines may be two subtypes of Th2 CD4+ T-cells, or may be

another cell type since other cell types known to produce these cytokines were also present in the culture milieu types (Gibbs *et al*. 1996, Shimizu *et al*. 1998). It would appear likely, however, that CD4+ T-cells produced the majority of IL-4 and IL-5 since it is generally accepted that T-cells are the major source of these cytokines (Jung *et al*. 1995, Karlen *et al*. 1998). The current results, which document an example of dissociated production of two Th2-type cytokines, underline the need to reconsider the classical Th1/Th2 paradigm.

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